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Vol. LVII *October, 1929* *No. 4*

BIOLOGICAL BULLETIN

THE REACTIONS OF *PARAMECIUM* TO SOLUTIONS OF KNOWN HYDROGEN ION CONCENTRATION.[1]

WILLIS HUGH JOHNSON,

THE UNIVERSITY OF CHICAGO AND WABASH COLLEGE.

INTRODUCTION.

The reactions of unicellular organisms to acids in dilute solution has been a subject for research for many years. Pfeffer in 1888 observed the positive reaction of spermatozoa to malic acid. Jennings ('97, '99*a*, '99*c*, '00*a*, '00*b*, '00*c*, '02, '06) described the attracting action of dilute acids for *Paramecium* and other protozoa. He expressed quantitative results in terms of fractional normality of acids rather than concentration of hydrogen ions. Garrey ('00), a student of Loeb, studying the reactions of *Chilomonas* to acids recognized the necessity for measuring the results in terms of hydrogen ion concentration but was able to do it only relatively rather than absolutely. Garrey and Jennings achieved results at variance and became involved in a lively controversy over the interpretations—a part of the larger Loeb-Jennings controversy. Greeley ('04) obtained positive reactions to dilute acid solutions with *Paramecia* taken from acid cultures. He states that he did not obtain positive reactions with *Paramecia* taken from alkaline cultures.

This field seems to have been abandoned by later workers. With the discovery of methods for determining the true hydrogen ion concentration of fluids and materials in recent years numerous investigations have been made concerning the part of acidity and alkalinity in biological processes, but none of these investigations have been along the lines followed by Jennings, Garrey

[1] The writer is greatly indebted to Dr. W. C. Allee for suggesting this investigation and for facilities and advice afforded in connection with it and to Mr. M. R. Garner for his coöperation in the initial experiments.

and Greeley. Later workers (Fine, '12, Collett, '19, Kostir, '21, Bodine, '21, Saunders, '24, Hopkins, '26, Pruthi, '27, Beers, '27, Eddy, '28, Darby, '29) have been more concerned with the tolerance of organisms to acids, the toxicity of acids in the media, the changes in natural acidity in protozoan cultures, and the effects of hydrogen ion concentration on different life processes.

No account of a repetition of the experiments performed by Jennings, Garrey and Greeley using modern methods of determining hydrogen ion concentration, as worked out by Sorensen and Clark and Lubs, has been found in the literature. The series of experiments described in this paper was undertaken to determine the reactions of *Paramecia* to acid solutions of known hydrogen ion concentration. The experiments were started during the summer of 1927 [1] in Whitman Laboratory and were continued there during the summers of 1928–29. During the winters of 1927–28 and 1928–29 the work was carried on at Wabash College, Crawfordsville, Indiana.

Methods and Materials.

A preliminary study was made to find satisfactory methods for carrying on the main series of experiments. Pfeffer (fide Loeb, '18) introduced the open end of a capillary tube sealed at the other end and filled with acid solution, into a drop of organisms. Loeb ('18) observes that Pfeffer's method of testing chemotropism is the best. Barratt (fide Loeb, '18) concluded, using Pfeffer's capillary tubes as described above, that *Paramecia* were indifferent to acid solutions. Barratt's experiments were repeated using HCl (pH 5.2) in capillary tubes and like results were obtained. When slightly larger tubes were used the organisms entered the acid in about ten times the numbers that entered the control. Ordinary pipettes were used with the result that seventy-five animals entered the acid while one entered the control. From these results it seemed evident that in the case of very small tubes it is almost impossible for a *Paramecium* swimming spirally to enter without striking the edge of the opening. And so Pfeffer's capillary tubes were not considered

[1] Mr. M. R. Garner and the present writer worked together on the first set of experiments to be described.

practical in these experiments. Jennings ('06) placed a cover slip, slightly elevated with glass rods, on a slide; introduced a drop of medium containing organisms on one side and acid solution on the other by means of a capillary pipette, or, merely placed a bit of acid at the edge of a cover slip under which the organisms lay. Garrey ('00) used a hollow cell for holding the organisms and introduced the acid through a small opening on one side. In addition to these methods two others were tried. (1) An acid gradient was made in a long tube (24 inches); the organisms were introduced at the end of the gradient corresponding in H-ion concentration to their own medium, and (2) a "double drop" method was devised in which a drop of medium containing *Paramecia* and a drop of acid solution were placed side by side on a slide and the two drops were connected by leading the liquids toward each other in a narrow line with the point of a needle. The former of these methods was discarded as being utterly impractical and the latter was chosen for use in preference to the older methods for a number of reasons. (1) Less elaborate apparatus was required. (2) The experiments were set up and carried on more quickly. (3) A more gradual gradient could be established than with the older methods.

Trays were made for holding the slides during the experiments. These trays, made by Mr. Carson in the shop at Whitman Laboratory, were fifteen inches in length, four inches in width and two and a half inches in depth with grooves on the sides to hold the slides in place. It was found that strips of black paper placed in the bottom of the trays aided greatly in making the counts at the beginning and end of each experiment.

The colorimetric method of determining hydrogen ion concentration was used except as noted. The standard tubes and indicators used were purchased from the La Motte Chemical Products Company or the Hynson, Westcott and Dunning Company, both of Baltimore, Md. The standard tubes covered a range from pH 3.0 to pH 8.4. The indicators were brom phenol blue, brom cresol green, methyl red, brom cresol purple and phenol red. The indicators, with the exception of methyl red, were made up in aqueous solutions. The exception was made up in an alcoholic solution as it is only slightly soluble

in water, but the quantity of alcohol introduced into a solution when this indicator was used was far below what Jennings found to elicit any sort of response. Both methyl red and brom cresol green were used in determining pH 5.0. Neither seemed to give very accurate and reliable results because the color change at this point is not very marked in the case of both. Hyman ('25) has noted this same difficulty with brom cresol green. Due to this difficulty it seems possible to explain some of the inconsistencies in the data at H-ion concentrations of pH 5.0.

Preliminary experiments were made to determine the degree of diffusion between the two drops. The usual result is illustrated in Fig. 1. Complete diffusion required about an hour while the

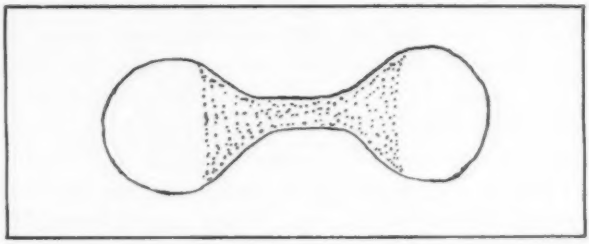

FIG. 1.

experiments usually ran about half an hour. The culture drops start drying first so any reading taken after half an hour would likely be affected by the drying in the culture drop. It was found that if the slides were too clean the drops spread rather badly. This could usually be remedied by rubbing one's hand over the slide to coat it with a film of oil. Experiments were carried on in duplicate, *i.e.* two slides bore the same drop combination. Experiments rendered unsatisfactory on account of the drops spreading or for other reasons were discarded. It was determined that it made no difference if the indicator was included in the acid solution.

Hay infusions were prepared by boiling 5 grams of timothy hay in 500 cc. of water for about five minutes. An additional 500 cc. of unboiled water was added to the infusion and after it had stood for a few days it was seeded with *Paramecia*. *Paramecium caudatum* as described by Wenrich ('28) were used.

The original seed was obtained from Dr. Peterson in Whitman Laboratory and all additional cultures were seeded from this strain. The cultures were not used until they were two or three weeks old. A new culture was prepared each week so that a number of cultures were on hand throughout the experiments. At first the cultures were kept in 1000 cc. Erlenmeyer flasks. It was found that the H-ion concentration of such cultures remained high for a long time. The cultures for practically all of the experiments were kept in wide-mouthed bottles and finger bowls. According to Jennings ('06), the fact that *Paramecia* collect in cultures in clumps where carbon dioxide is present often causes misleading results, so in all of the experiments the *Paramecia* were stirred and aërated to eliminate this factor.

The following acids were used: hydrochloric, nitric, sulphuric, carbonic, formic, acetic, citric, picric, pyrogallic, and tannic. Carbonic acid was prepared by bubbling CO_2 into water until the desired H-ion concentration was obtained. As the CO_2 soon escapes into the air and thus changes the H-ion concentration, it seemed necessary to find a way of covering the drops when carbonic acid was used. This was done by cementing glass strips on the edges of ordinary slides. The tray holding these "chamber slides" was placed in a large covered pan and CO_2 was allowed to escape into the pan. Then the drops were placed in the "chamber slides" and connected as quickly as possible. As soon as the drops were connected each "chamber slide" was covered with an ordinary slide. Indicators showed that the change in H-ion concentration from the time the drop was placed on the slide until it was covered was not great.

The following different kinds of water were used in making up the acid solutions: ordinary Chicago tap water, Hull[1] well water, Whitman[2] well water, Crawfordsville (Ind.) tap water, distilled water, Chicago tap water made carbonate-free, Whitman well water made carbonate-free and Hull well water made carbonate-free. The carbonate-free water was made according to the method used by Hyman ('25). Two cc. of concentrated

[1] From Hull Zoölogical Laboratory.

[2] From Whitman Experimental Laboratory.

hydrochloric acid were used to eight liters of water in a large bottle. Air from the compressed air system was passed through the water in the bottle for at least 24 hours. Analyses of the Whitman well water and the Hull well water will appear later in the paper.

At first there was a question as to what constituted a positive reaction. The constant moving about of the *Paramecia* made it appear that some of the crossing over from one drop to another must be attributed to random movements on the part of the *Paramecia*. It was thought that the absence of *Paramecia* from one drop might be a factor in causing the crossing over. A set-up was made to test this idea. Some culture liquid from a culture with a concentration of pH 7.7 was centrifuged. The culture fluid, free from *Paramecia*, was drawn off and used in an experiment in the same way that acidified water had been used—a drop of the culture fluid free from *Paramecia* was joined to a drop of culture containing *Paramecia*. There was no change in the H-ion concentration from centrifuging. Table I gives the result of this experiment. At the time of reading which was that used in other experiments about equal numbers were found in each drop. On the basis of these results, reactions of over 55 per cent were considered as positive reactions. This indicates that the absence of *Paramecia* from one of the drops is not a factor in causing the positive reactions.

TABLE I.

SHOWING AMOUNT OF RANDOM MOVEMENT ON PART OF *Paramecia*.

	pH 7.7 (Centrifuged Culture liquid)					
	45	55	50	51	46	48
	46	46	56	60	51	73
	53	58	41	49	56	52
	36	61	40	40	53	43
	56	56				26/1326
Ave. % reaction.....						51

A number of experiments were undertaken during the Christmas holidays (1928–29) but not many were successful. The temperature of the laboratory during this time ranged from 15° C.

to 21° C. The only experiments which gave any positive results were conducted when the temperature was 21° C. The experiments run when the temperature was lower than 21° C. gave no positive results. Jennings ('06) states that Mendelssohn found that the optimum temperature for *Paramecium* is between 24 and 28 degrees C. All of the experiments recorded here were conducted within a temperature range of 21 to 28 degrees C. No data were obtained as to the upper temperature limit for positive reactions.

Experiments.

(1) *To Determine pH Range in which Positive Reactions Occur.*

In the beginning several experiments were carried out using a pH range of 3.2 through 5.8. It became evident, after using several acids and repeating the experiments, that no positive reaction (it was considered that the *Paramecia* must enter the acid drop) could be obtained at a H-ion concentration greater than pH 5.0 with any of the acids used. Any H-ion concentration of pH 4.8 or lower proved to be toxic to *Paramecium*. So with the lower limit for positive reactions established, the next problem was to determine the reactions of *Paramecium* to a range of H-ion concentrations of pH 4.8–5.8. The following table shows the results obtained with hydrochloric, sulphuric and nitric acids in Chicago tap water.

Section *A* of this table shows the results of six set-ups. Two slides with the same H-ion concentration were used in each set-up, as the table shows. The numbers in the different columns represent the percentage reaction on each slide from which the mean average reaction was obtained. The number of *Paramecia* on each slide averaged about fifty. This was true for all of the experiments. According to the criterion established above Table II. shows positive reactions from pH 5.0 through pH 5.8 in the three inorganic acids used. There seemed to be no significant difference in the reactions with the different acids. The H-ion concentration of the cultures used in these experiments ranged from pH 7.4 to pH 8.0. Controls (in duplicate) were run in each set-up by joining a drop of culture with a drop of unaltered tap water. No positive reaction occurred in any of the controls. The high percentage reaction (81 per cent.) at

TABLE II.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CHICAGO TAP WATER.

(Data in section *A* obtained in conjunction with Mr. M. R. Garner.)

A (1927).

	pH 4.8	5.0	5.2	5.4	5.6	5.8
	10	73	96	84	80	22
Hydrochloric	12	84	70	73	88	25
	25	18	70	90	67	78
Hydrochloric	10	27	92	80	50	79
	23	23	95	70	72	76
Sulphuric	4	42	89	71	70	69
	41	56	59	54	43	74
Sulphuric	58	87	74	55	39	18
	60	68	87	55	56	61
Nitric	45	88	65	65	60	51
	22	65	82	75	52	50
Nitric	58	56	92	59	83	58
	12/368	12/686	12/971	12/829	12/760	12/661
Ave. % reactions	30	57	81	69	63	55

B (1928).

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	35	73	53	67	80	91	
Hydrochloric	70	83	54	59	89	94	40
	23	41	43	47	91	70	
Hydrochloric	50	47	77	37	97	78	6
	44	65	54	80	23	30	
Hydrochloric	20	63	95	30	56	63	9
	36	53	81	70	74	71	
Hydrochloric	52	76	92	70	100	97	26
	44	65	92	54	72	98	
Sulphuric	60	40	80	51	78	75	41
	41	72	67	97	67	67	
Sulphuric	58	50	83	54	63	72	9
	9	70	74	77	60	69	
Sulphuric	34	74	70	54	50	85	30
	15	90	85	72	83	64	
Sulphuric	17	80	77	72	77	61	21
	13	70	67	82	70	60	
Nitric	22	81	67	82	52	87	34
	18	50	73	62	80	77	
Nitric	20	82	70	71	80	65	0
	28	82	50	74	88	70	
Nitric	29	80	60	81	80	68	8
	22/738	22/1487	22/1564	22/1463	22/1610	22/1611	11/224
Ave. % reactions	33	67	71	66	73	73	20

pH 5.2 seemed to be a possible optimum but later experiments did not indicate such a marked reaction to any one H-ion concentration. In all of the experiments the temperature of the acid solutions was maintained the same as the temperature of the culture used.

The results in Section *B* are very similar to those listed in Section *A*. One difference seems to be that there is no marked optimum in the reaction given at any pH as was the case in the reactions to a concentration of pH 5.2 in Section *A*. The figures in the column under pH 7.8 show the results in the controls which were like the controls used in the first series of experiments. The cultures used ranged in H-ion concentration from pH 7.4 through pH 7.8. Another difference is evident in that the percentage of reactions at pH 5.8 in the second series is no lower than at any other pH in the range used. Thus it became necessary to extend the range of experimentation. A greater range had not been used in the first series because time would not permit.

Several organic acids were used over the same pH range. Acetic, citric and formic acids elicited responses somewhat comparable to those of the inorganic acids as shown in Table II. Pyrogallic and tannic acids proved to have such a high degree of toxicity for the animals that reactions within the pH range studied were impossible—death resulting on contact with the weakest part of the diffusion area. This high degree of toxicity of tannic acid may offer an explanation of the absence of Protozoa from bog waters. Picric acid was used several times with inconsistent results. The strong color of the acid solution prevented accurate pH readings. It also seemed that here, as with pyrogallic and tannic acids, there is some other factor besides the H-ion concentration which affects the reactions of *Paramecia*.

Later in the summer of 1927 cultures with higher H-ion concentrations (pH 6.8, pH 7.0 and pH 7.2) were used and no positive results were obtained at the above ranges. Several times throughout the period of experimentation cultures with these concentrations were used with the same results—no positive reactions. When the H-ion concentration of the cultures fell, positive results were obtained.

Table III. shows that positive reactions can be obtained in a pH range of 6.0–6.8, which, taken with the results of the two other series, means that *Paramecia* from alkaline cultures react positively throughout a pH range of 5.0–6.8. No positive response was obtained in the controls at pH 7.8.

TABLE III.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 6.0–6.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CHICAGO TAP WATER.

	pH 6.0	6.2	6.4	6.6	6.8	7.8
	44	78	88	57	79	
Hydrochloric	62	66	66	65	54	38
	54	84	81	61	76	
Hydrochloric	83	85	55	70	77	2
	40	40	62	80	63	
Sulphuric	53	40	70	58	80	0
	51	48	51	58	62	
Sulphuric	33	51	36	52	57	38
	89	77	81	77	57	
Nitric	80	43	73	70	60	21
	46	48	46	43	56	
Nitric	57	49	54	50	46	30
	46	81	64	59	69	
Nitric	76	75	54	65	76	0
	14/814	14/865	14/881	14/865	14/914	7/129
Ave. % reactions	58	62	63	62	65	18

A series of experiments were run using Hull well water. The following table gives the results when unaltered Hull well water was acidified with nitric, hydrochloric and sulphuric acids.

The cultures used in Table IV. had a H-ion concentration of pH 7.8. Unaltered Hull well water, pH 7.8, was used in the controls. These data show that *Paramecia* from cultures with a low H-ion concentration (pH 7.8) react about the same way to acidified Hull well water as they react to acidified Chicago tap water.

All the data seem to point rather conclusively to the fact that *Paramecium caudatum* as reared in these cultures do not react positively to H-ion concentrations greater than pH 5.0. As one of the objects of this problem was to determine the pH range for positive reactions, it seemed necessary at this point to run more experiments attempting to determine the upper limit

for positive responses. Crawfordsville (Ind.) tap water, coming from a number of surface springs, was used. This water always had a H-ion concentration of pH 7.4. Table V. gives the results of a series of experiments using unaltered Crawfordsville tap water, acidified with hydrochloric, sulphuric and nitric acids, over a pH range of 5.0 to 7.2. The unaltered tap water, pH 7.4, was used in the series as a control. The cultures used in this series had H-ion concentrations of pH 7.6 to pH 7.8.

TABLE IV.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN HULL WELL WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	9	72	90	85	79	79	
Hydrochloric	23	80	79	70	71	89	47
	20	36	60	90	93	70	
Sulphuric	15	20	50	83	79	65	43
	10	40	70	84	80	79	
Nitric	17	30	81	89	89	76	26
	6/94	6/278	6/430	6/501	6/489	6/458	3/116
Ave. % reactions	16	46	72	83	81	76	38

It will be noticed by examining Table V. that positive reactions were obtained throughout the entire range used—pH 5.0–7.2. And not only were positive reactions obtained throughout this range, pH 5.0–7.2, in which the H-ion concentration was produced by adding acid to the water, but positive reactions were also obtained in the controls, unaltered tap water, with a H-ion concentration of pH 7.4. If all of the preceding tables are examined it will be found that in the other controls the H-ion concentration in each case was the same as, or lower than, the H-ion concentration of the culture used. In this case the H-ion concentration of the control used was higher than the hydrogen ion concentration of the cultures used. This pointed to another set of experiments using water with a H-ion concentration the same as the culture used. All of the controls of the preceding experiments indicate that *Paramecium* will not react positively to a solution with a H-ion concentration the same as, or lower than, the H-ion concentration of the culture. Indicators showed

TABLE V.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecium* TO HYDROGEN ION CONCENTRATIONS AT pH 5.0–7.2 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CRAWFORDSVILLE TAP WATER.

	pH 5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4
	44	74	83	67	87	80	75	71	74	48	79	64	73
HCl	52	65	62	70	81	81	75	74	53	70	83	83	70
	43	68	63	86	82	81	84	70	87	74	74	91	59
HCl	70	76	85	84	86	69	78	79	80	75	72	81	69
	84	75	88	78	92	69	80	72	69	72	89	75	78
HCl	63	70	85	88	81	76	64	79	85	71	86	78	73
	51	57	72	76	81	80	65	69	67	78	73	73	76
HCl	57	70	80	64	70	75	59	75	69	62	78	72	65
	54	67	80	100	86	78	86	88	85	84	89	88	58
HCl	62	80	54	86	65	57	74	79	93	71	75	88	56
	33	55	71	83	74	72	83	56	65	82	73	84	69
HCl	31	47	68	84	74	81	77	62	89	81	80	74	58
	27	43	78	81	77	92	79	89	83	84	79	89	63
H_2SO_4	32	48	86	79	84	89	88	72	87	81	88	87	56
	62	60	81	76	77	79	80	68	77	80	80	83	82
H_2SO_4	55	77	73	72	70	75	82	74	75	77	79	75	78
	30	53	62	65	74	69	75	60	50	54	49	57	61
H_2SO_4	48	24	68	50	79	75	69	60	53	40	70	46	83
	24	92	86	91	91	92	100	89	82	81	86	83	77
H_2SO_4	25	91	84	87	89	91	87	89	66	80	77	86	83
	26	41	32	67	84	50	62	52	40	31	60	84	83
H_2SO_4	27	44	32	60	62	41	55	44	46	41	66	79	87
	45	47	75	60	75	60	71	87	72	70	76	50	61
H_2SO_4	59	63	72	61	51	78	75	66	72	74	50	56	73
	64	83	90	90	76	79	94	35	75	88	70	79	84
HNO_3	53	79	81	76	81	84	85	85	72	89	90	88	82
	41	53	61	53	79	70	77	84	88	81	51	53	79
HNO_3	53	56	60	80	53	69	80	83	91	85	54	82	59
	26	57	60	58	71	44	40	75	55	53	70	76	33
HNO_3	20	56	63	61	74	46	52	63	51	67	89	64	41
	59	46	81	80	71	69	73	79	63	74	86	67	76
HNO_3	55	49	74	89	79	85	75	79	74	71	77	76	80
	56	43	60	93	85	76	41	66	64	82	50	65	64
HNO_3	58	54	93	84	70	60	38	84	73	81	68	65	70
Ave. % reactions	34/1592 47	34/2063 61	34/2443 72	34/2574 76	34/2601 76	34/2470 72	34/2478 72	34/2455 72	34/2435 71	34/2432 71	34/2516 74	34/2541 74	34/2359 69

that the H-ion concentration of the drops made from Crawfordsville tap water fell as soon as the drops were exposed to the air, so it was possible by aërating the water to lower the H-ion concentration to pH 7.8. Table VI. gives the results of the set of experiments using *Paramecia* from a culture with a concentration of pH 7.8 and Crawfordsville tap water aërated so that the concentration was pH 7.8. A control was used by testing the reaction of *Paramecia* to a concentration of pH 5.6 made by adding nitric acid to Crawfordsville tap water. Positive reactions were obtained in the controls but not in the drops where the H-ion concentration of the two drops was the same. This would seem to indicate the *Paramecia* from cultures with a low H-ion concentration react positively to any H-ion concentration higher than that of their culture to a pH of 5.0, which is the lower limit for positive reactions.

TABLE VI.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO A HYDROGEN ION CONCENTRATION OF PH 7.8 MADE BY AËRATING CRAWFORDSVILLE TAP WATER.

	5.6 (Nitric Acid).	7.8 (Aërated Crawfordsville Tap Water).				
	75	11	12	35	44	31
	82	4	18	40	28	41
	61	27	16	24	33	27
	78	38	27	24	36	11
Ave. % reactions..	4/296 74	36	41			22/639 29

(2) *To Determine Whether the Positive Reactions are Due to Carbon Dioxide or to Hydrogen Ions.*

Jennings ('06) suggests that the positive reactions of *Paramecia* to dilute acids is closely connected with their natural tendency to collect in clumps when a large amount of carbon dioxide is present. Hyman ('25) found that "the depressing action of acids in natural waters is due chiefly or wholly to the carbon dioxide which they liberate from the carbonates of such waters." With these ideas in mind a series of experiments was run in an attempt to determine whether the *Paramecia* react to carbon dioxide or to the free hydrogen ions. Twelve sets (like

the sets described in the preceding tables) were run from pH 4.8–6.8 with carbonate-free water made from Whitman well water using hydrochloric, sulphuric and nitric acids with no positive results. I was ready to conclude from these results that *Paramecia* react to carbon dioxide and not to free hydrogen ions when I realized that Whitman well water had not been used in any of the preceding experiments. So twelve more sets were run over the same range using Whitman well water as it comes from the tap and acidified with the same acids. These results were also negative. As a result the use of Whitman well water had to be discontinued. I can give no reason for the negative results. Analyses of Whitman well water, as well as of Hull well water with which positive results were obtained, were made but the differences were not great. No presence of B. coli was found in Whitman well water; so it seems that the unfavorable reactions to this water must be due to obscure chemical causes.

Some Hull well water was then made carbonate free in the manner already described. The carbonate-free water had a pH of 4.6. The pH was raised to 7.8 by adding NaOH. The water was then acidified. Table VII. shows the results of a series of experiments using carbonate-free Hull well water acidified with hydrochloric, sulphuric, and nitric acids.

TABLE VII.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CARBONATE-FREE HULL WELL WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	48	67	53	55	89	51	7
Hydrochloric	40	40	78	70	87	57	34
	39	34	55	70	83	75	3
Sulphuric	60	65	54	92	81	77	3
	51	66	54	56	86	70	27
Nitric	30	60	54	75	94	73	23
	6/268	6/332	6/348	6/418	6/520	6/412	6/97
Ave. % reactions	45	55	58	69	86	69	16

The cultures used in the above experiments had *a* concentration of pH 7.6. Carbonate-free Hull well water with a sufficient amount of sodium hydroxide to make the pH 7.8 was

used in the controls. This series of experiments indicated that the *Paramecia* were reacting to the free hydrogen ions and not to the carbon dioxide.

The reaction of *Paramecia* to Hull carbonate-free well water acidified with carbonic acid was tried. The carbonate-free water was lowered to pH 7.8 with sodium hydroxide. Carbon dioxide was then passed through the water until the desired acidity (pH 5.6) was obtained. It was found that carbonic acid elicited a response similar to that elicited by the three inorganic acids. The experiment was carried out in the manner described earlier in the paper. Table VIII. shows the results. The cultures used had H-ion concentrations of pH 7.7 and pH 7.8. Carbonate-free water with sodium hydroxide added to it giving a H-ion concentration of pH 7.8 was used in the controls.

TABLE VIII.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO A HYDROGEN ION CONCENTRATION OF pH 5.6 MADE WITH CARBONIC ACID IN CARBONATE-FREE HULL WELL WATER.

	pH 5.6	7.8
	76	40
Carbonic acid	71	20
" "	94	25
" "	88	25
" "	76	0
" "	73	20
" "	75	45
" "	50	20
" "	73	11
" "	12	21
" "	44	37
" "	64	10
" "	89	4
" "	70	3
" "	78	20
" "	67	9
" "	83	11
" "	70	21
	18/1253	18/342
Ave. % reactions	70	19

Chicago tap-water was made carbonate-free in the same manner and used in a series of experiments. The Chicago carbonate-free tap-water tested pH 4.6 when it was made, and, as in the experiments with Hull carbonate-free well water, the H-ion concentration was lowered to pH 7.8 by adding sodium

hydroxide. Table IX. gives the results of a series of experiments using Chicago carbonate-free tap-water. The cultures used tested pH 7.8. Carbonate-free water plus sodium hydroxide with a H-ion concentration of pH 7.8 was used in the controls. The results are like those obtained in the other experiments over this pH range except in the cases where Whitman well water was used. These results also indicate that the *Paramecia* react to free hydrogen ions and not to carbon dioxide.

TABLE IX.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CARBONATE-FREE CHICAGO TAP-WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	53	86	52	72	86	86	21
Hydrochloric.....	55	33	88	61	76	63	46
	46	63	74	40	83	65	21
Sulphuric.....	30	64	76	83	81	46	42
	42	44	61	85	86	66	22
Nitric.....	20	41	62	77	75	64	16
	6/246	6/331	6/413	6/418	6/487	6/390	6/168
Ave. % reactions....	41	55	69	69	81	65	28

(3) *To Determine Reactions of Paramecia to Distilled Water.*

Two sets of experiments were run using distilled water. The distilled water tested pH 5.6 and the *Paramecia* reacted to it positively. However, in order to run a series of experiments over the pH range 4.8 to 5.8, the H-ion concentration of the distilled water was lowered to pH 7.8 by adding NaOH. The water was then acidified to obtain the desired H-ion concentrations. The cultures used had a H-ion concentration of pH 7.7–7.8. Distilled water plus NaOH with a H-ion concentration of pH 7.8 was used in the controls. Table X. gives the results of two sets of experiments using distilled water altered with NaOH. Positive results were obtained but these results differed from any of the other results obtained using inorganic acids in this respect; in all of the drops the *Paramecia* were dead, or at least becoming inactive, at the time of the reading.

TABLE X.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC AND NITRIC ACIDS IN DISTILLED WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	13	67	65	56	61	100	10
Hydrochloric.....	60	54	60	42	63	62	7
Nitric.....	25	82	77	63	75	54	27
	37	88	69	77	60	76	25
	4/135	4/291	4/261	4/238	4/259	4/292	4/69
Ave. % reactions....	34	73	65	60	65	73	17

(4) *To Determine pH at the End of the Experiments.*

It was evident that the H-ion concentration in the drops fell during the duration of the experiments. An attempt was made to determine the H-ion concentration at the end of the experiments. The sulphonphthalein indicators apparently are not harmful to *Paramecia* and do not affect their reactions when no greater concentration of the indicator is used than is necessary for making a pH reading in one of the tubes. Indicators were added to the drops used in the experiments. The results of many observations are given in Table XI. It is very difficult to make pH readings of the drops at the end of the experiment in this way. The density of the color in the drops is much less than the density of the color in the tube when the pH reading is determined. If enough of the indicator is added so that the density of the color in the drops is like that in the standard tubes, it is impossible to make accurate pH readings of the liquid to start with. So these observations recorded in Table XI. must be considered only as approximations. The writer is not convinced that the H-ion concentration at the end of the experiment is of very great importance because in many cases quite positive reactions occurred before the H-ion concentration had changed to any appreciable degree.

(5) *To Compare Determinations Made with Indicators and with Potentiometer.*

All of the H-ion concentration determinations which have been listed above were made using the colorimetric method. An

additional series of experiments was run in which the H-ion concentrations were determined by both the colorimetric method and the electrometric method for the sake of comparison. A

TABLE XI.

SHOWING pH AT START AND END OF EXPERIMENTS.

pH at Start of Experiment.	pH at End of Experiment.
5.0	5.8 to 6.2
5.2	6.4 to 6.6
5.4	6.8 to 7.2
5.6	7.2
5.8	7.4
6.0	7.4
6.2	7.6
6.4	7.6
6.6	7.6
6.8	7.6
7.0	7.8
7.2	7.8
7.4	7.8

Youden hydrogen ion concentration apparatus, manufactured by the W. M. Welch Co. of Chicago, was used in making the electrometric determinations. The H-ion concentrations determined with the potentiometer were consistently lower than those made using the color indicators, showing a difference in pH of .15 to .26 with a mean difference of .21. The reactions of *Paramecium*

TABLE XII.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 5.0–7.6 MADE WITH NITRIC ACID IN CHICAGO TAP-WATER AND THE DIFFERENCE IN HYDROGEN ION CONCENTRATIONS WHEN MEASURED WITH BOTH COLOR INDICATORS AND POTENTIOMETER.

pH Color.	pH Potentiometer.	Difference.	Reactions.
5.0	4.97	0.21	56
5.2	5.00	0.20	73
5.4	5.16	0.24	81
5.6	5.38	0.22	81
5.8	5.62	0.18	79
6.0	5.79	0.21	79
6.2	5.96	0.24	78
6.4	6.21	0.19	77
6.6	6.38	0.22	71
6.8	6.65	0.15	74
7.0	6.79	0.21	78
7.2	7.03	0.17	77
7.4	7.20	0.20	73
7.6	7.36	0.24	66
7.8 (tap water)	7.55	0.25	31
7.7 (culture)	7.44	0.26	

were about the same as those in the other experiments. The following table will show the results. The figures listed under the column "pH Potentiometer" represent the average of three determinations. The figures under the column "Difference" represent the average difference of the three determinations. The figures under the column "Reactions" represent the average reactions of five observations at each H-ion concentration.

It should be stated that the above comparison does not affect the results of the other experiments. This comparison merely shows that the pH range is moved back approximately 0.21 when the H-ion concentration is determined with the potentiometer used in this case. The almost identical mean determinations (4.97 and 5.00) with the potentiometer indicates the difficulty mentioned before in determining H-ion concentrations of pH 5.0 and pH 5.2 with the color indicators.

Discussion.

Jennings ('06) says that "specimens in water that is decidedly alkaline collect even more readily in acids than do those in a neutral fluid." This was found to be the case in these experiments except that *Paramecia* from slightly acid and neutral cultures did not give positive responses according to the method used in reckoning positive responses. Greely ('04) states that only *Paramecia* from acid cultures react positively to acids. This does not agree with Jennings. There is nothing in these experiments which is in agreement with this statement; in fact I have found the exact opposite to be true.

Child and Deviney ('26), Pruthi ('27) and many others have mentioned the different stages that occur in the life history of a protozoan culture. Four distinct stages were noticed in the cultures of *Paramecia* used in these experiments. The first stage was not very long in duration except in cases when Erlenmeyer flasks were used, and the H-ion concentration was high (pH 6.8–7.0). The second stage was marked by a steady fall in H-ion concentration. In the third stage the H-ion concentration was low and remained fairly constant. In this stage the *Paramecia* were scattered throughout the culture. Most of the *Paramecia* used in these experiments were in this stage. The

last stage was marked by the *Paramecia* gathering in the bottom of the culture and dying out. *Paramecia* from cultures in the last stage were never used in the experiments because it seemed that the toxic substances in such a culture, as well as the general physiological state of the *Paramecia*, might prevent normal reactions.

An examination of all of the data shows that *Paramecium* (from cultures with a low H-ion concentration) react positively to all H-ion concentrations higher than that of their culture to a concentration of pH 5.0 inclusive. This limit for positive reactions is the same as Crane ('21) found to be the highest H-ion concentration in which *Paramecium* can live 24 hours. I have checked this observation and have obtained essentially the same results.

Close observations demonstrate rather clearly that *Paramecia* give the same response upon coming into contact with strong acids (more acid than pH 5.0) and upon coming into contact with their old culture medium after they are already in the acid solution (less acid than pH 5.0). In both cases they give the avoiding reaction.

Dr. L. G. Barth in his Doctor's Dissertation,[1] "The Effect of Acids and Alkalies on the Viscosity of Protoplasm," has found in unfertilized *Arbacia* eggs that acids if they are able to penetrate coagulate at pH 5.0. This suggests a reason why *Paramecia* are not able to live in concentrations of acids lower than pH 5.0.

Incidentally some observations were made on the toxicity of acid solutions. Pruthi ('27) observed that animals from a culture with a concentration of pH 7.8 placed in a hydrochloric acid solution with a concentration of pH 6.0 died within half an hour. Throughout these experiments it was observed that the organisms were in good condition after being in even stronger acids for considerable periods. Some *Paramecia* from a culture with a concentration of pH 7.8 were stirred into a citric acid solution with a concentration of pH 5.7. At the end of a half hour and an hour and a half some were placed back in culture media. In each case the animals seemed to suffer no ill effects and reproduced in an apparently normal manner. Again some *Paramecia*

[1] Placed in the University of Chicago Library in 1929. To be published later.

were placed in a hydrochloric acid solution with a concentration of pH 6.0 and left. Three days later the H-ion concentration had fallen to pH 6.2 and the animals were in normal condition. Pruthi also observed that *Paramecia* reproduce more rapidly in their own culture medium than in tap-water of the same H-ion concentration and suggests that this may be due to a greater amount of carbonates in the culture medium. To the writer of this paper it seems that a much more definite factor is food, as rate of division is closely correlated to amounts of food available.

Jennings' ('06) statement that "*Paramecia* collect in all weakly acid solutions, no matter what acid substance is present," was confirmed in these experiments. In the cases of pyrogallic acid and tannic acid no positive responses were obtained at the H-ion concentration used. To make solutions of these two acids with a pH range of 4.8–5.8, almost saturated solutions were necessary. It seems that some other factor besides the H-ion concentration is responsible for the great toxicity of these two acids. However, it was interesting to note that *Paramecia* reacted to weaker solutions of these two acids. In each case when pyrogallic or tannic acid was used the *Paramecia* gathered in the neck between the two drops and died there. This bears out another statement by Jennings to the effect that there is no relationship between the attracting or repelling power of acids and their injurious effects. Since it was apparent that all acids elicited the same responses, unless they were toxic in the pH range used, the three inorganic acids, hydrochloric, sulphuric and nitric acids, were used in most of the experiments in order that a more concentrated study might be made.

It is well known that carbon dioxide plays an important part in the behavior of *Paramecia* in natural conditions. The work of Hyman on Planaria ('25) made it appear that *Paramecia*, in reacting to acidified water, might be reacting to the carbon dioxide liberated from the carbonates instead of to free hydrogen ions. The series of experiments using carbonate-free water were run with the aim of finding out something in this connection. Although the writer realizes that many biologists today feel that too much significance has been ascribed to H-ion concentration "per se" in biological reactions, the results of the experiments

using carbonate-free water give a strong indication that *Paramecia*, when reacting to weakly acid solutions, react to the free hydrogen ions. This idea does not seem to be out of line with the natural conditions that cause *Paramecia* to gather in clumps where carbon dioxide is present. The carbon dioxide in the cultures where *Paramecia* gather in clumps is in the form of carbonic acid.

Jennings ('06) has noted that all alkalies, save the alums, have a strong repellent effect on *Paramecia*. The observations made in these experiments that *Paramecia* will not react positively to a H-ion concentration lower than that of their culture tend to support this conclusion. The repellent effect of alkalies on *Paramecia* seems to help explain the fact that *Paramecia*, in cultures which have become quite alkaline (so far as is known the change in H-ion concentration in cultures cannot be regulated by the *Paramecia*), react to solutions with a H-ion concentration higher than that of their cultures.

Loeb ('18) based his conclusion that *Paramecia* are indifferent to acid solutions on Barratt's experiments using Pfeffer's capillary tubes. In repeating Barratt's work it seemed evident that the size of the openings in the tubes is the factor which explains the indifferent reactions. *Paramecia* cannot enter the small tubes without striking the edges. They can and will enter larger tubes containing weakly acid solutions.

The nature of the response of *Paramecia* to acid solutions received some consideration also. Jennings ('99) believed that the aggregations resulted from trap action, *i.e.* from a series of negative reactions to solutions of low acidity when the animals were in acid, rather than from a true positive chemotropism. Garrey ('02) working with *Chilomonas* concluded that the organisms showed positive chemotropism to acetic, lactic, and butyric acids, but that aggregations in inorganic acids resulted from trap action or "chemokinesis" as he called it. His conclusion was made possible by the manner of orientation and slow movement of the organisms he studied. The rapid movement of *Paramecia* through diffusion areas prevents accurate conclusions as to the nature of the responses to acids, so it was not possible to distinguish between the two types of reaction

as observed by Garrey. It seemed evident, in these experiments, that the *Paramecia* reached the acid region by trial or random movements, and that once within the acid trap action occurred. Crossing back and forth between drops which had stood a long time was often noticed but in such cases the indicators would usually show that complete diffusion had taken place throughout both drops.

In the experiments when carbonate-free water or distilled water was used NaOH was added to lower the H-ion concentration in each case to pH 7.8. In all of these experiments the *Paramecia* tended to gather in clumps in a much more striking way than they did in any of the other experiments. In some cases the clumping occurred in the neck between the two drops, making it necessary to discard such experiments. In these experiments hydrochloric, sulphuric, nitric and carbonic acids were added to the water, altered with NaOH to produce the desired H-ion concentrations. As a result NaCl, Na_2SO_4, $NaNO_3$ or Na_2CO_3 was formed in the solutions. Jennings ('06) has listed NaCl, $NaNO_3$ and Na_2CO_3 as being very repellent to *Paramecia*. He further states that when a repellent substance, as NaCl, is mixed with an acid and a drop of the mixture is placed on a slide of *Paramecia* the *Paramecia* gather in a ring about the drop. In these experiments it seems that the attracting power of the H-ions is greater than the repellent power of the salts formed.

One of the most puzzling features of the whole series of experiments was the failure of the *Paramecia* to react positively to Whitman well water. Although analyses of Whitman well water and Hull well water were obtained for reference, no possible cause for the difference in eliciting positive responses can be given at this time. The *Paramecia* seem to serve as very delicate indicators in this connection, and the fact that *Paramecia* do not react positively to Whitman well water may be of importance in future experimentation with this water.

Summary.

1. The reactions of *Paramecium caudatum* to solutions of known hydrogen ion concentration were studied.

2. The colorimetric method of determining hydrogen ion

concentration was used but was checked by a series determined both colorimetrically and by Youden's potentiometer.

3. The acids used were: hydrochloric, nitric, sulphuric, carbonic, formic, acetic, citric, picric, pyrogallic and tannic.

4. The waters used were: Chicago tap-water, Whitman well water, Hull well water, Crawfordsville (Ind.) tap-water, distilled water, Chicago tap-water made carbonate-free, Whitman well water made carbonate-free, and Hull well water made carbonate-free.

5. In these experiments only *Paramecia* from alkaline cultures responded positively to acidified solutions.

6. *Paramecia* from alkaline cultures react positively to all H-ion concentrations higher than that of their culture to pH 5.0 (4.83 potentiometer reading) inclusive, when the H-ion concentration is produced by the inorganic acids—hydrochloric, sulphuric and nitric acids.

7. Organic acids, such as acetic, formic, carbonic and citric, which are not toxic in this pH range elicit responses similar to those caused by the inorganic acids.

8. Pyrogallic and tannic acids are toxic to *Paramecia* in solutions with concentrations of pH 4.8 through pH 5.8 but the organisms enter these waters and die as a result.

9. *Paramecia* react to acidified water from which all carbonates have been previously removed. This is an indication that *Paramecia* react to the hydrogen ion "per se" and not to carbon dioxide.

10. *Paramecia* are better able to live in solutions of high H-ion concentration than many have supposed.

11. Sulphonphthalein indicators, when used in no stronger concentration than necessary for pH determinations, are not harmful to *Paramecia*.

12. *Paramecia* will not react positively to weakly acid solutions when the temperature is below 21 degrees C.

13. No new evidence was obtained as to the nature of the mechanics of the reaction of *Paramecia* to acids.

14. *Paramecia* gather in clumps when they react to acidified solutions containing NaOH.

15. In one of the four types of water tested acidified water did

not elicit positive responses from *Paramecia*. No reason is offered for this exception.

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REGENERATION IN *POLYCHŒRUS CAUDATUS* MARK.[1]

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INTRODUCTION.

The purpose of these observations was to re-examine and supplement the investigations of Stevens (1905), and Child (1907) on the regeneration in the acœlous flat worm—*Polychœrus caudatus*.

The observations and experiments on living material were carried out at the Marine Biological Laboratory, Woods Hole, during the summers of 1927, 1928, 1929.

HISTORICAL

Polychœrus caudatus was given its name by Mark in a paper which contains an excellent description of the external and internal anatomy (1892). Gardiner published papers on, "The early development of *Polychœrus*" (1895), and "The growth of the ovum, formation of the polar bodies, and the fertilization in *Polychœrus caudatus*" (1898). Löhner in 1910 examined Mark's description and added a more detailed account of the nervous system.

Regeneration in *Polychœrus* was first described by Stevens and Boring (1905)—Miss Stevens doing the experiments on the living material and Miss Boring the histology of regeneration. Miss Stevens sectioned the worms by two general type cuts, that is, by transverse cuts at two levels of the body, and also by longitudinal oblique cuts. She observed regeneration in almost all these pieces, the only exception being a failure of anterior regeneration in some of the "middle pieces." Miss Boring concluded that regeneration in *Polychœrus* was by morphallaxis.

[1] I wish to express my sincere appreciation to Professor J. Walter Wilson whose helpful suggestions made these investigations possible; and to Dr. William A. Castle who also read the manuscript.

Child (1907) gave a more detailed and extensive account of regeneration. Using the statocyst as a landmark in locating the cephalic ganglion, he made transverse, longitudinal and oblique cuts. In the transverse cuts he noted that "posterior regulation does not occur in the preganglionic region." As the transverse cuts were made progressing more posteriorly toward the caudal lobes, the amount of posterior regeneration gradually decreased and redifferentiation increased. Anterior regulation in pieces anterior to the ganglia was mostly by redifferentiation, while directly posterior to the ganglia it was by regeneration. As the level of cut proceeded toward the caudal lobes, anterior regeneration gradually decreased until at levels in the posterior fourth of the animal, it was limited to wound closure. In observing lateral regulation, Child found that the 'half' animal without the statocyst never regenerated its lost half, while the 'half' containing the statocyst showed complete regeneration. Oblique regulation proved to be a combination of lateral and longitudinal regulation. Child considered these facts to show that a relation existed between the nervous system and "form regulation" which appeared to be functional.

Material and Methods.

About six hundred specimens were used in this study of regeneration. They were collected in Little Harbor at Woods Hole, where at low tide great numbers are easily found on the green Ulva.

In the laboratory they were kept in large shallow dishes in a cool shaded place, the sea water being changed every other day. No attempt was made to feed the worms, and therefore they decreased gradually in size through lack of food.

The individual animals were isolated by means of wide-mouthed pipettes. To operate, the worms were transferred into a Syracuse watch glass which had been filled with paraffin. All the operations were performed with a sharp scalpel under the binocular microscope. The different series of operated animals were then placed in labeled Stender dishes, and daily observations were made.

In order to record and measure the cuts with respect to the

position of the statocyst, it was necessary to isolate the operated animal upon a glass slide (under low power) and gently press the cover slip. The pressure was controlled by vaseline, and only in this way could the writer see the statocyst. It appears somewhat like a small air bubble and can be easily missed.

Figures were drawn from the living experimental animals as accurately as possible.

Experimental Observations.

In the following discussion of experimental observations, the author is using Morgan's terminology for the restoration of parts. "Morphallaxis" is interpreted as the remolding of old tissue directly into a new form without a proliferation at the cut surface. Parts are replaced by "epimorphosis" when there is a "proliferation of material preceding the development of the new part" (1901, p. 23), that is, an outgrowth of new tissue from which the missing part may develop. Morgan includes both of these processes under the single word "regeneration" which he uses to signify the restoration of form. Child uses the term "regulation" as parallel to Morgan's "regeneration." This "regulation" may occur either by "redifferentiation" of old tissue (Morgan's morphallaxis) or by "regeneration" (Morgan's epimorphosis).

In a number of the following experiments, the type of cut has been made in relation to the position of the main ganglionic masses. A short account of the nervous system, therefore, seems necessary.

Mark's anatomical description of the nervous system shows "a pair of ganglionic masses connected by a transverse commisure which lies above the otocyst" (p. 304). From these ganglionic masses nerve trunks arise. Mark considers the chief nerves of this type of system rather comparable to that described in *Convoluta* by Délage (1886) and von Graff (1891).

In 1910 Löhner, a student of von Graff, made an extensive histological study of *Polychœrus*, including a detailed account of the nervous system. He found that *Polychœrus* had one median unpaired ganglion, and two lateral ganglia symmetrically placed —that is, one on either side of the median ganglion, approximately

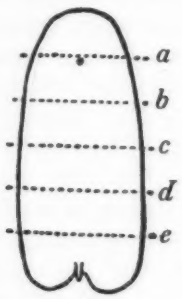
mid-way between the median ganglion and the lateral edge of the animal. The statocyst he found to be imbedded in the median ganglion (see Löhner's diagram on Plate 17, or Hanström, p. 91).

In this paper the author is following Löhner's description of the nervous system and therefore, the use of the words "main ganglionic masses" refers to the three ganglia as described above.

The first experiments were to re-examine the type cuts made by Child, and to supplement with slight modification.

Posterior and Anterior Regeneration.

Transverse sections were made at five different levels as indicated in Fig. 1, and regeneration at each level was observed. Posterior and anterior regeneration was noticed to agree with Child's observations (1907) at comparable levels. That is, if a



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worm is cut in front of the ganglia, the anterior piece neither regenerates new caudal lobes by epimorphosis nor reorganizes to a normal form.

If a worm is cut posterior to the ganglia at levels preceding toward the caudal lobes—the amount of posterior epimorphosis decreases and reorganization increases as the level of cut reaches the posterior end.

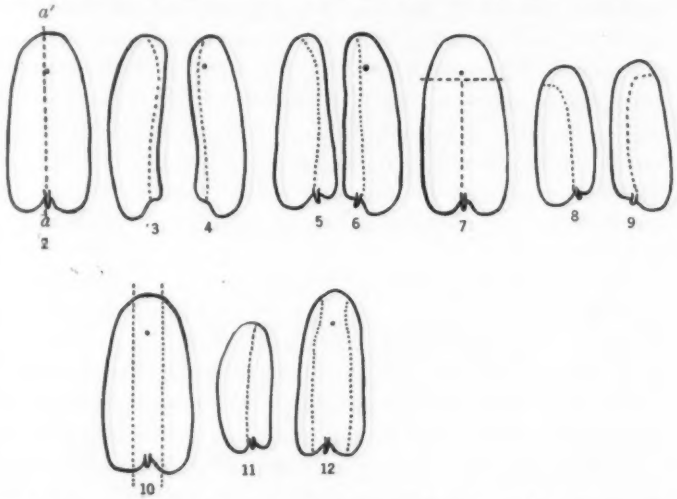
The posterior piece of a worm that had been sectioned anterior to the ganglia, shows complete regeneration by morphallaxis rather than by epimorphosis. At all levels posterior to the

ganglia, anterior regeneration was never complete—the amount of epimorphosis decreasing as the cuts preceded towards the caudal lobes.

Lateral Regeneration.

It was in lateral regeneration that a difference was noted between Child's experiments (1907) and those recorded in this paper.

Sections were made along *a a'* (Fig. 2) and the amounts and rates of regeneration in the two halves were compared. Immedi-



ately after the operation, the worm contracted on the side toward the cut surface, thereafter moving in a circle. Ten days after sectioning the pieces without the statocyst appeared to have regenerated just as much as did the pieces containing the statocyst (Fig. 3, 4) and movement was not in a circular direction. After twenty days, the "half" without the statocyst and median ganglionic mass, had formed a distinct caudal lobe, and the amount of new tissue was almost equal to that in the other "half" (Fig. 5, 6). It is surprising to note that the "half" without the statocyst behaved at first as a "headless piece," but as might be expected, when regenerated tissue appeared, the behavior of the two "halves" did not differ when mechanically stimulated.

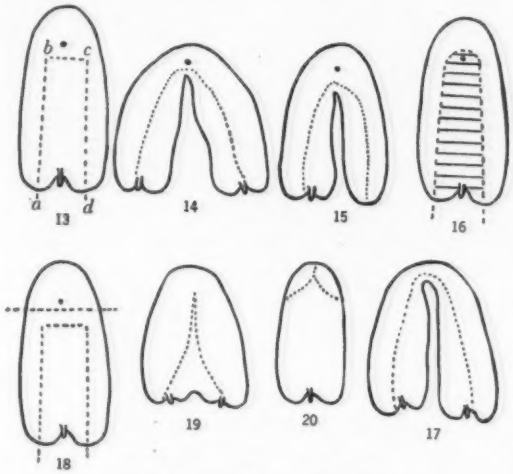
A combination of lateral and anterior regeneration was then made (Fig. 7), to compare with the above lateral regeneration. Seven days after the operation, all the pieces regenerated both anteriorly and laterally (Fig. 8, 9).

The results of sectioning as indicated in Fig. 10, dividing the animal into three parts, showed regeneration in the following manner. Within six days each marginal piece had formed by epimorphosis a new caudal lobe, while the old tissue at the posterior margin extended posteriorly and reorganized, thus replacing the other lobe (Fig. 11). As might be expected, no statocyst could be identified in either marginal piece, although the animals were more active than the so-called 'headless piece' produced by anterior cuts. The middle pieces, containing the statocysts, did not regenerate as rapidly as did the marginal pieces—the anterior part of the animal showing the more rapid lateral regeneration. Within two weeks, however, the middle pieces, by epimorphosis and morphallaxis appeared almost normal (Fig. 12).

Some modifications of lateral cuts were made as shown in Fig. 13. The animals were cut along line *a b c d*, leaving the statocyst in place. Regeneration was very rapid, taking place within five days. Each marginal piece regenerated laterally, and formed a caudal lobe with filaments as in Fig. 14. The animal had the appearance of two animals with one head, but moved as one. In one instance, the 'one animal' did not develop any caudal lobes, thus giving an asymmetrical form (Fig. 15).

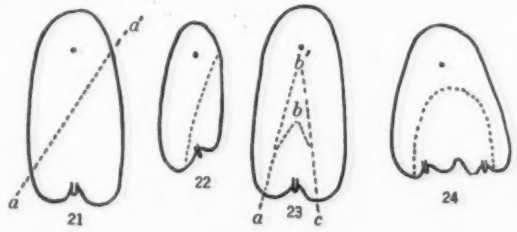
Sections were then made (Fig. 16) to find if there were lateral regeneration in the absence of the median ganglionic mass. Apparently there was no obvious correlation, for regeneration was neither retarded nor modified. If "they" had not pulled apart, then the *Polychærus* appeared as in Fig. 17, five days after the operation. However, sections cut as in Fig. 18, showed slight variations. Due to the fact that these worms were less active, the greater part of the lateral cut surfaces healed, and the animal appeared as in Fig. 19. The fate of that portion of the animal indicated by cross hatching in Fig. 16, was also observed. These pieces did not regenerate beyond wound closure either laterally or anteriorly, and as a result of their inability to regenerate, the

animals often showed a partial union of the cut edges at the anterior portion. The caudal tissue maintained its function of attachment (Fig. 20).



OBLIQUE REGENERATION.

Animals were sectioned along *a a'* (Fig. 21). The results were as might be expected from combinations of lateral and longitudinal regeneration. The posterior marginal edge reorganized into a right caudal lobe, while the left one was formed by epimorphosis (Fig. 22).



Some combinations of oblique cuts gave interesting results. If the animals were sectioned along *a b c* (Fig. 23), the anterior part of the animal regenerated two pairs of caudal lobes with tail filaments (Fig. 24).

Similar results are observed later as in Fig. 26. The more anterior the cut, the greater the amount of lateral replacement. If the section removed extended to just behind the statocyst (*a b' c*, Fig. 23), the amount of lateral replacement was so great as to give the appearance of a form shown by Fig. 14.

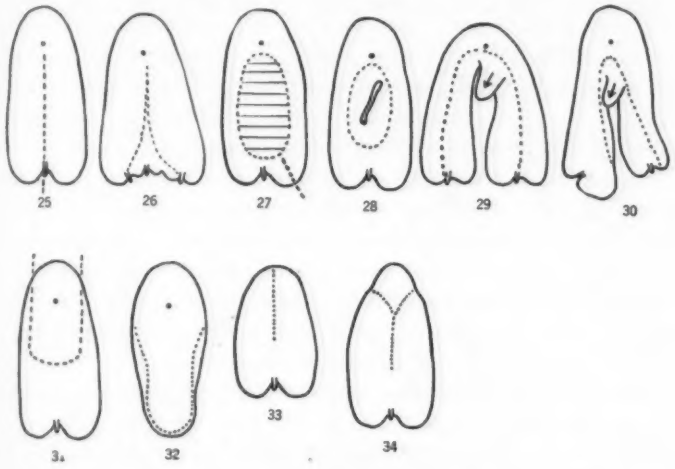
Regeneration Following Other Types of Cuts.

Longitudinal incisions were made through the caudal lobes and extending anteriorly to the statocyst—thus dividing the animal posteriorly into two parts (Fig. 25). *Polychærus* showed remarkable powers of healing. Within an hour the animal healed almost the entire length of the cut, a small portion of the caudal part of the incision remaining open. The result of this cut was that two or three pairs of caudal lobes often appeared where one pair was normally present. Tail filaments appeared at the caudal notches (Fig. 26).

The same type of incision was made at the anterior end. The animals healed within an hour and behaved normally. No obvious morphallaxis nor epimorphosis was noted in these anterior cuts.

When large circular pieces from the center of the worm were removed leaving a "living ring" (Fig. 27), new tissue rapidly regenerated from the cut surface, and replaced the tissue removed. If the wound did not close within four days, the regenerated tissue never closed the opening. In such cases, an open slit was left until death (Fig. 28). If the circular piece was not quite as large as in Fig. 27, the animal again displayed its remarkable healing and reorganizing powers. The wounded surface was drawn together, and within seven to ten days by morphallaxis and epimorphosis, the animal assumed its normal appearance. Fig. 29–30 show two unusual results from animals that had been cut as is indicated in Fig. 27. The "living ring" was broken at the region of the caudal notch (shown by dotted lines in Fig. 27), and by nine days the lateral regeneration of two pairs of caudal lobes with filaments, had taken place. A week later, a new growth of tissue appeared at the notch, which extended and pulled posteriorly as a head might at such a location. The animal lived about a month after the operation, and up to that

time, no statocyst had developed in this regenerated 'head.' These observations recall rather similar results in *Planaria* observed by Lemon (1899), Van Duyne (1896), Bardeen (1901) and Morgan (1900) which show the formation of a head in the notch of the cut. Many animals were cut similarly in hopes of obtaining more of the same results, but to the time of writing, no more have occurred.



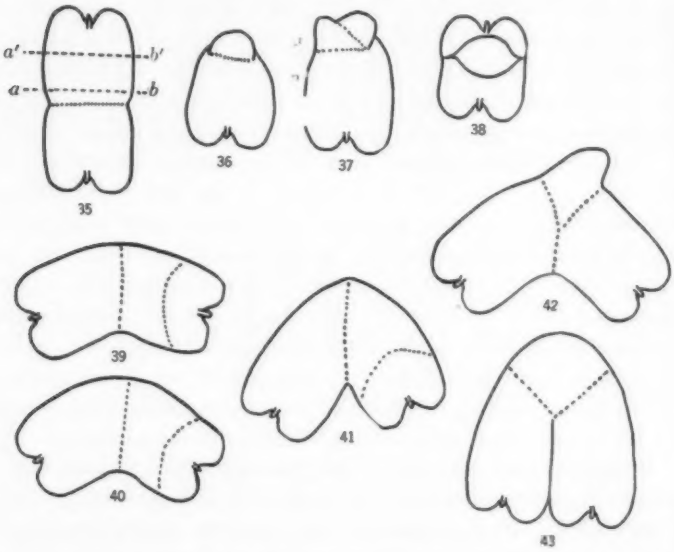
If the animals were cut as shown in Fig. 31, the anterior portion formed normal lobes and filaments by lateral and posterior regeneration. In one instance (Fig. 32) there was regeneration of the missing lateral part, but no posterior regeneration beyond wound closure. The posterior part of the cut worm was also observed. Within an hour the worm showed wound closure by drawing the cut surfaces together (Fig. 33). Ten days later, regenerated tissue began to push out gradually (Fig. 34). About two weeks later the animal assumed its normal form again, although no new statocyst was ever observed. In one or two instances regeneration did not occur. The worm remained as Fig. 33 and showed marked thickening until death.

Experimental Grafting.

The rapid healing ability noted in *Polychærus*, suggested the possibility of successful grafts and the regeneration of grafted pieces.

The object of these grafting experiments was to study the possibility of reversal of polarity in *Polychærus*.

Lillian Morgan's technique used for uniting desired pieces of *Planarians* (*Planaria maculata* and *Phagocata gracilis*) was tried



unsuccessfully on *Polychærus*. It appeared that *Polychærus* needed more water than was offered by moist filter paper. The 'chance method' of union, that is, cutting the worms in two, and placing together about forty of the posterior pieces in a salt cellar, gave the best results. In about 16 per cent. grafts were obtained in which two pieces were united by their anterior cut surfaces (Fig. 35). These grafted pieces were then isolated into separate dishes. The union was quite perfect, the region of union almost indistinguishable and usually no regeneration occurred. The compound animal, however, always behaved as two separate individuals, and slight locomotion was possible

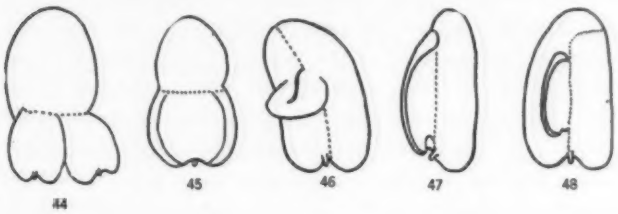
only when one individual lost its hold upon the substrate. It then might be pushed about by the other piece.

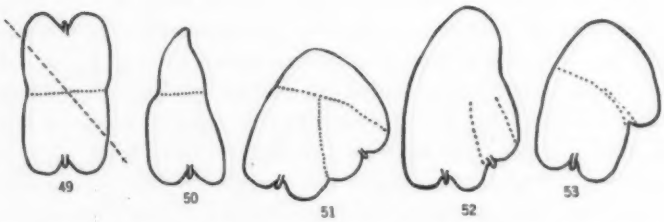
Ten grafts were cut in two, at a level very near the region of union of the two pieces (lines *a b*, Fig. 35). Eight of these, neither regenerated beyond wound closure at the cut surface, nor regenerated at the region of union. The smaller grafted pieces rounded up, showing marked dorsal thickening (Fig. 36) The pieces were watched for twenty days. Of the remaining two, one piece regenerated at the region of union within three days after the cut. The "rounded up" tissue appeared to be pushed to the side, as the larger piece regenerated its anterior tissue (Fig. 37). The other piece showed regeneration of caudal lobes and a tail filament at the cut surface (Fig. 38).

In another experiment, twelve grafted compound animals were cut at a different level (*a' b'*, Fig. 35), farther away from the region of union. In every instance caudal lobes and filaments were formed. A slight locomotion was gradually accomplished by the worms assuming positions as shown by Fig. 39, 40, 41. In three instances the compound animals not only regenerated the lost lobes, but also "heads" at the region of union (in this "head" no statocyst could be identified). This "head" did not seem to belong especially to either component part, but pushed out from between the pieces (Fig. 42, 43). These experiments with reverse grafted pieces recall Lillian Morgan's (1906) experiments with reverse grafted pieces of *Planaria* in which a head formed from the line of union; and Joest's experiments with *Allolobophora terrestris*, when he grafted together the anterior ends of two worms, and one or two heads developed at the region of union.

In some of these grafts, before the second cut was made on the compound animal, the dorsal surface of the one animal was continuous with the ventral surface of the other (one animal was on its back when the graft formed). It was found that either the caudal lobes of one component were used for attachment; or both caudal lobes attached themselves to the substrate by their ventral surfaces. In the later instance, the animal was twisted at the region of union, the twist assuming the form of a bulge.

Two other combinations of pieces were formed by the "chance method." Fig. 44 shows two small posterior pieces attached to the posterior portion of a larger anterior piece. In Fig. 45, a posterior part is shown attached to an anterior piece, the dorsal surface of one animal continuous with the ventral surface of the other. They remained in this form until death.





In one instance a graft occurred in a cut like that described in Fig. 10. The union between the middle and right marginal piece, with a different orientation, resulted in a form as shown in Fig. 46. The larger piece regenerated laterally while the smaller grafted piece seemed to be gradually absorbed by the larger.

Another example of absorption of the smaller grafted piece is shown by Fig. 47, 48. This graft occurred between the two lateral pieces of a worm cut as shown in Fig. 7. The graft was such that ventral and dorsal surfaces were continuous. The larger piece regenerated laterally and anteriorly and therefore pushed the smaller graft on its dorsal side. A gradual absorption of the smaller piece occurred.

In another series of graft experiments, the anterior ends of the animals were united, and later the pieces were cut obliquely

through the line of union (Fig. 49). Twenty-four hours after, the small grafted piece still functioned as a caudal piece, although the cut surfaces contracted thus giving the appearance of Fig. 50. It was difficult to distinguish the union of the graft in the contracted worm. Ten days later, new caudal lobes and a filament had developed in a number of animals, and the worms assumed the relative position shown by Fig. 51. Whether the caudal lobes developed entirely from the small graft or from the larger piece, could not be detected (Fig. 51, 52). (A difficulty of the same sort is mentioned by Morgan in similar cuts on grafted *Bipalium kewense*.)

In a few cases the cut oblique portions healed together and no regeneration occurred beyond wound closure, thus giving the appearance as in Fig. 53.

A series of oblique regeneration from grafted pieces was made, in which one piece of the graft was vitally stained with Nile Blue Sulphate. It was hoped that in this way the exact origin of new tissue (Fig. 51–52) could be determined. In this respect the results of this experiment were unsuccessful for two reasons—*i.e.*, the stain had to be of such a dilute nature in order to be vital, that the detection of the dye in the regenerated portion was not apparent, or the stained portion of the graft was sluffed off before regeneration could occur.

Attempts were made to graft two anterior ends by their posterior cut surfaces. The pieces, however, crawled away from each other so that all attempts of this kind proved unsuccessful.

Discussion.

From Child's experiments (1907) and the authors' observations it would seem evident that the absence of the median and lateral ganglion prevent complete anterior and posterior regeneration.

The difference in rate and method of locomotion between animals with and without the ganglia was noticeable for some time after the operation. This time may be from five to ten days. The 'headless' pieces at first appeared more sluggish than the others, but within two weeks they behaved quite normally—provided that dorsal thickening had not taken place.

It appears that lateral regeneration occurs in the absence of the

median ganglionic mass (Fig. 3, 4, 5, 7). This seems to agree with L. V. Morgan's observations in *Leptoplana littoralis* (1905, p. 193). Since it is thought that the lateral ganglion and likewise the nerve roots near the median ganglion still remain in *Polychœrus* when sectioned as in Fig. 3 (as Child suggested with reference to *Leptoplana littoralis*—1910, p. 338), it might seem that their presence might influence lateral regeneration. But in comparing modifications of lateral cuts (Fig. 16, 18) it again indicates that in *Polychœrus*, the median and lateral ganglia themselves do not play such an essential rôle in regeneration as would be expected. In the experiments reported in this paper, the worms regenerated always into a normal form, although regeneration was retarded slightly and the statocyst was never obviously replaced.

In recent correspondence with Dr. Child concerning this apparent disagreement between his observations and mine, that is, regeneration from pieces of *Polychœrus* cut in the median lateral plane without the median ganglion (Child's Fig. 31), Dr. Child offers several possible interpretations:

First, a species difference. The species of *Polychœrus* studied at Pacific Grove, California, and that at Woods Hole, Massachusetts, may not be the same and ". . . differ slightly as regards the ganglionic distribution."

Second. The longitudinal cuts described by Child may be somewhat more lateral than those described in this paper, and therefore the amount of the ganglionic masses would be different.

Third. The pieces of *Polychœrus* in lateral regeneration as described by Child were not observed as long as other pieces. Dr. Child writes, "I believe that these particular pieces were not followed as long as some others and that they might perhaps later have shown a change and come to resemble more or less closely those which you describe."

Fourth. ". . . the possibility that differences in physiological age and development might account for differences of this sort."

With respect to these differences Dr. Child remarks, "in any case the difference seems to me of minor importance and to indicate that some difference in material or experimental conditions had been involved in the two cases."

As the longitudinal cuts approached the margin of the animal, epimorphosis decreased and morphallaxis increased. This confirms Child's theory of functional substitution, as stated in the historical summary of this paper.

Whenever the marginal posterior part of the animal is separated from its other margin, two or more caudal lobes are formed (Fig. 14, 15, 17, 24, 29, 30). This recalls the work of T. H. Morgan (1900) and of Randolph (1897), who produced double tails in *Planaria* by oblique cuts.

By reviewing the cuts which gave rise to the two tailed worms (Fig. 13, 16, 18, 23, 25) it appears that the length of the regenerated posterior region may be controlled by the depth or length of the cut and by the amount of wound closure. Note in Fig. 14 and 17 that the cuts extended far anteriorly and separated the marginal parts of the animals. These cuts gave rise to two distinct posterior parts, that is, four caudal lobes with a filament in the two notches. Cuts which did not extend so far anteriorly and showed more wound closure, resulted in a smaller amount of posterior regeneration (Fig. 19, 24, 26).

Miss Stevens observed in the "posterior regulation" of a few "middle pieces" a regeneration of supernumerary appendages and caudal lobes. In repeating these experiments similar results have been obtained only when the cut separated the posterior region. (The writer is lead to think that the supernumerary appendages described by Miss Stevens in her Fig. N, were due to a slight wound in these middle pieces caused when they were "violently disturbed," p. 339.) Miss Stevens referring to these extra appendages remarks that, "all the variations observed in regeneration are to be found in normal adult worms" (p. 337). Such a statement appears startling, for in the hundreds of normal worms observed, the writer has seen only three or four individuals that showed more than the normal number of lobes and appendages, but by close observation it was seen that a wound had formed and regeneration had taken place before the worms had been brought into the laboratory, thus giving the animal three or four lobes with four or five filaments. Mark in his description of the species says, "I have never seen an individual with more than three tail filaments" (p. 301), nor does Child

mention such extra normal tail filaments in his account of regeneration.

The rapid wound closure taking place in animals that had been cut by a circular incision (Fig. 27), agrees with Child's remark that in *Turbellaria* and in many other forms "nutritive and other conditions are better in such a cleft, where the growing parts are in contact on both sides with other tissue, than on surfaces where such contact exists only on one side" (1910, p. 334). When the cuts were circular (Fig. 27), if neither the cut surfaces of the worm fused together nor the regenerated tissue closed the opening within four or five days, then the slit remained open as long as observations was continued (Fig. 28). It appears that the muscles about the open slit, do not continue to contract after new tissue had been formed for three or four days. The muscles then relax as in a normal regeneration of tissue.

Instances similar to the two where head formation occurred in the angles between the posterior parts (Fig. 29, 30), were never observed again. Cuts indicated by Fig. 13, 23, 25, 27 were expected to give similar results. (When the cut surfaces healed immediately, head formation was not expected.) Van Duyne, Lemon, Bardeen and Morgan easily obtained similar heads in *Planaria* by making longitudinal incisions in the tail, forward to about the level of the eye spots. Such a head formation in *Polychærus* should not be mistaken for a true heteromorphic head. Morgan unlike Van Duyne, does not interpret such heads in *Planaria* as heteromorphic heads—"the new heads develop in connection with the new material of the half, and appear at the side as does the new head in longitudinal pieces, and are, therefore, not, in one sense, heteromorphic productions" (1900, p. 98).

In grafting pieces of *Polychærus*, so that the posterior ends were united by their anterior cut surfaces, it was noted that when grafts were cut at a level near to the region of union, in most cases the smaller grafted pieces failed to regenerate caudal obes, and rounded up by extreme dorsal thickening. In the other type cut, in which the cut was farther from the region of union, caudal lobes and filaments always regenerated. Therefore, in both these types of regeneration from grafted pieces, no

reversal of polarity occurred. This seems to agree with L. Morgan's experiment with pieces from the middle region of *Planaria* which had been reversed and grafted to the head region of another.

Child suggests that in *Polychærus* there exists a relation between the central nervous system and form regulation. In my experiments the prevention or delay of regeneration in the one type of grafted cut (Fig. 35 *a b*), may then be due to the fact that the grafted piece is very small and therefore contains a smaller amount of the longitudinal nerve trunks than it does in the other type of graft cut (Fig. 35 *a' b'*).

Since grafts did not occur between extreme anterior ends of *Polychærus*, the regeneration of reversed pieces from the head region could not be watched. Whether the polarity of the longer piece might have influenced the smaller grafted piece (Fig. 36, 37, 51, 52, 53), as it appeared to do in L. Morgan's experiment with *Planaria*, still remains to be shown.

The appearance of a head in some instances as the region of union (Fig. 37, 42) and its lack of appearance at other times, might indicate that the cut surfaces of the graft were not united their entire length. This would occur if the worms were of a different size. In the case shown by Fig. 37, the regenerated head seemed to originate from the larger piece, and it assumed the position of the larger pieces' head. However, in the case shown by Fig. 42, the head seemed not to belong specifically to either graft. The head continued to grow larger and morphallaxis seemed to occur in both the grafts—the head becoming as broad as both component grafts (Fig. 43).

Summary and Conclusions.

1. If a worm is cut in front of the ganglia, the anterior piece neither regenerates new caudal lobes by epimorphosis nor reorganizes to a normal form.

2. If a worm is cut posterior to the ganglia at levels preceding toward the caudal lobes—the amount of posterior epimorphosis decreases and reorganization increases as the level of cut reaches the posterior end.

3. The posterior piece of a worm that had been sectioned

anterior to the ganglia, shows complete regeneration by morphallaxis rather than by epimorphosis. At all levels posterior to the ganglia, anterior regeneration was never complete—the amount of epimorphosis decreasing as the cuts proceeded towards the caudal lobes.

4. If a worm is cut longitudinally in half, lateral regeneration occurs in both the 'halves' with and without the median ganglionic mass (Fig. 3, 5).

5. The difference in locomotion between worms with and without the median ganglion and statocyst, is noticeable within ten days after the cut. The pieces without the sense organ act as 'headless pieces' showing difficulty in righting themselves when overturned, and moving only when mechanically stimulated. As regeneration occurred, these 'headless pieces' gradually approached the normal type in behavior.

6. In another experiment, the anterior end was cut off, and then the rest of the worm cut longitudinally in half. The absence of both median and lateral ganglia does not prevent lateral-anterior regeneration (Fig. 3, 5, 6, 7, 8, 9).

7. If a worm is cut as in Fig. 10 giving two narrow pieces from the sides, regeneration takes place and a small symmetrical worm is formed (Fig. 11). The middle piece forms mostly by reorganization into the normal form (Fig. 12).

8. If the posterior part of an animal is cut in two longitudinally, or cut so that the marginal regions are separated from each other and the wound does not heal, each half or part produces new tissue and forms distinct caudal lobes and filaments. The length of the posterior regenerated region may be controlled by the depth of the cut, or removal of the middle tissue and wound closure. The absence of this middle part does not seem to hinder regeneration (Fig. 14, 17, 18, 24, 26).

9. If the region of the median ganglion is removed, a slight retardation of regeneration occurs, but lateral regeneration is not impaired (Fig. 16).

10. If the cut surfaces do not heal together from a circular cut, or the regenerated tissue does not close the wound within four or five days, then the slit remains open until death (Fig. 28).

11. If the posterior part of a worm is cut as far forward as the

statocyst (Fig. 29, 30), head formation may appear in the notch between the new sides.

12. Reversed grafts between anterior surfaces of *Polychœrus* are readily obtained.

13. If the line of union in reversed grafts is perfect, no regeneration takes place, and locomotion is only possible when one component part of the grafted animal looses its hold on the substrate.

14. When reversed grafts are not perfectly united a head develops from the exposed part. This head may or may not appear to belong to either piece (Fig. 37, 42).

15. If a very small piece is grafted to a larger piece and regeneration occurs in the larger piece but not in the smaller, then absorption of the smaller piece takes place (Fig. 46, 47, 48).

16. When pieces which had grafted together by anterior ends were cut obliquely, the grafted pieces showed morphallaxis and caudal lobes developed from the cut surface (Fig. 51, 52); except in a few cases (Fig. 53). Just which part of the graft regenerated the caudal lobes is not certain, since the detection of the vital dye in the graft was not apparent.

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A STUDY OF EQUILIBRIUM IN THE SMOOTH DOGFISH (*GALEUS CANIS* MITCHILL) AFTER REMOVAL OF DIFFERENT PARTS OF THE BRAIN.

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In the experiments here reported we have studied equilibrium after removal of

1. End-brain
2. Optic lobes
3. Cerebellum
4. End-brain, optic lobes and cerebellum

In the teleosts, Desmoulius, Vulpian, Flourens and Steiner detected no disturbance of equilibrium after removal of the end-brain. Polimanti noticed that removal of the end-brain caused cessation of motility; even if stimulated with electrical current the animals failed to move. When locomotion did take place it was hesitant and uncertain; equilibrium was disturbed. In the selachians, Steiner found that removal of the end-brain caused immobility for hours and even days. Bethe and Polimanti observed that the animals remained active; equilibrium remained normal.

Baudelot and Vulpian failed to detect disturbances of equilibrium in the teleosts after removal of the roof of the mid-brain. In the selachians, Steiner, Bethe, Loeb obtained similar results; after removal of the optic lobes equilibrium remained normal. Contrary to Baudelot, Vulpian, Steiner, Bethe and Loeb, Polimanti found that removal of the optic lobes in the teleosts and selachians was followed by disturbances of equilibrium.

Renzi and Dickinson observed profound disturbances of equilibrium in the teleosts after removal of the cerebellum (hind-brain). These authors believed the cerebellum to be indis-

pensable for equilibrium. Vulpian and Polimanti noticed that equilibrium remained normal after removal of the cerebellum. In the selachians, Steiner, Bethe, Loeb agreed that removal of the cerebellum produces disturbances of equilibrium. Polimanti's observations concerning the removal of the cerebellum in the selachians did not permit him to deduce conclusions; the results were not decisive. Elsberg and Tilney have found no disturbance of equilibrium after removal of the cerebellum; equilibrium in every respect was comparable to the equilibrium of the normal animal.

Our experiments have been restricted to the selachian (*Galeus Canis*). The size of the animal varied from 50 cm. to 75 cm. Special care was taken to operate while the gills and the anterior portion of the animal's head remained submerged in sea water. The animal was fastened to a cork board inclined at an angle of about 35° in a large dissecting pan. The pan was provided with a continuous flow of sea water.

The different regions of the brain were approached through the roof of the brain case (tegmen cranii). Making first a skin flap, the portion of the cartilaginous encasement above the region to be ablated was removed. After ablation of the particular region, the parts of the brain that remained exposed were covered with cotton. The skin flap was replaced and sutured. All animals died from two to three days after having been operated upon.

The end-brain (cerebral hemispheres and olfactory lobes) was removed by making an incision at the junction (velum transversum) of this part of the brain with the inter-brain (thalamus). Care was taken to injure neither the optic nerves nor the chiasma.

The cerebellum was removed by cutting through its peduncles —the superior and inferior peduncles.

The optic lobes were removed by cutting around the region of each optocœle; to avoid injuring the floor of the mid-brain, precaution was taken not to extend the ablation beyond the optocœles.

Studies of equilibrium were made in an aquarium $2\frac{1}{2}$ meters in length, $1\frac{1}{4}$ meters in width, 4/5 meter in depth.

DATA.

Removal of the End-Brain.—No disturbance of equilibrium resulted from removal of the end-brain. The animal swam normally in all planes; at the bottom of the aquarium it settled dorsal side up. Placed on its back at the surface of the water, the animal righted itself immediately. Equilibrium was comparable to the equilibrium of the normal animal. Six animals were operated upon.

Removal of the Optic Lobes.—Eight animals were operated upon. In each animal both optic lobes were removed. In three cases the animal swam normally in all planes; in five cases it frequently swam indiscriminately to the right or to the left around the dorso-ventral axis. This disturbance of movement in the horizontal plane cannot be attributed to partial loss of equilibrium because changing the direction of the animal changed the direction of its swimming; turned to the right the animal swam to the right around the dorso-ventral axis; turned to the left, it swam to the left. In these latter cases, movement in the horizontal plane became normal within twenty-four hours after removal of the optic lobes; the tendency to swim around the dorso-ventral axis disappeared. In all eight cases, the animal settled dorsal side up at the bottom of the aquarium. Placed on its back at the surface of the water, the animal righted itself immediately. It failed to avoid the sides of the aquarium.

Removal of the Cerebellum.—The cerebellum was removed in seven animals. The ablation caused no disturbance of equilibrium; the animal swam normally in all planes and settled dorsal side up at the bottom of the aquarium. There was complete absence of rotation around the axes, spirals through the water, nose diving and swimming on its back. Placed ventral side up at the surface of the water the animal righted itself without difficulty; no disturbance of equilibrium was noticed when righting itself.

Removal of the end-brain, optic lobes and cerebellum: Eight animals were operated upon. In each animal the end-brain, the optic lobes and cerebellum were removed. The operative procedure was as follows:

1. The end-brain was removed.
2. The animal was placed in the aquarium for two hours.
3. The optic lobes were removed.
4. Four hours after removal of the optic lobes, the cerebellum was removed in those cases in which movement in the horizontal plane remained normal after removal of the lobes; in the cases in which removal of the optic lobes caused the animal to swim around the dorso-ventral axis, the cerebellum was removed after movement in the horizontal plane had become normal.

No disturbance of equilibrium resulted after completion of all operations. The animal swam normally in all planes but did not avoid the sides of the aquarium. Rotation around the axes, spirals, nose diving and swimming on its back did not occur. The animal was active. Placed on its back at the surface of the water it righted itself immediately and without difficulty. At the bottom of the aquarium it settled dorsal side up.

Conclusion.

In the smooth dogfish, *Galeus Canis*, removal of the end-brain, the optic lobes or the cerebellum does not effect disturbances of equilibrium; equilibrium remains normal even after these three parts of the brain have been removed in the same animal.

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HETEROAGGLUTINATION OF DISSOCIATED SPONGE CELLS.

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It has been shown in my previous papers (Galtsoff, 1923, 1925) that in a suspension of artificially separated tissue cells of *Microciona prolifera* the amœboid movement of the archæocytes is instrumental in the formation of cell aggregates and that the movement of the archæocytes is inhibited by the presence of the cells of another species. The inhibitive effect of the foreign cells is apparently due to the substances excreted by them or, more probably, liberated from those foreign cells which were injured in the preparation of the suspension.

Further observations disclosed the fact that when in a compound suspension of *Microciona* and *Cliona* the archæocytes of two sponges come together, the outer hyaline layers of their protoplasms fail to coalesce, the cells of each species remaining separated and forming aggregates of its own kind. There is no doubt that such a segregation is due to the difference in the physical properties of the outer protoplasmic layers of the cells of two species. It was the author's intention to continue a study of this phenomenon on a greater variety of sponges. Thanks to the generous grant of the American Association for the Advancement of Science, the author was able to carry out the experiments at the Bermuda Biological Station in June–July 1925. The publication of the results has been delayed on account of the difficulty of identifying some of the Bermuda sponges and because of other investigations in which the author was engaged. It is the privilege of the author to express his thanks to the A. A. A. S. for a grant which made this work possible; to Dr. E. L. Mark, Director of the Bermuda Biological Station, for the permission to occupy a laboratory space; and to Dr. H. V. Brøndsted, Birkerod, Denmark, for the identification of six species of sponges.

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Material and Method.

All the sponges used in the experiments were collected in the shallow water around Agar's Island. Previous experiments with *Microciona* and *Cliona* had shown that sponges do not keep well in laboratory aquaria and that after a few days of confinement their regenerative ability is greatly reduced. Because of the necessity of having an abundant supply of fresh material always at hand the selection of species has been governed by their abundance and accessibility. The following siliceous and horny sponges were used:

Reniera cinerea Grant; *Pachychalina* sp.; *Spinosella sororia* Dendy; *Tedania ignis* D. and M.; *Donatia* sp.; *Suberites carnosus* Johnston; *Aplysina crassa* Hyatt; *Aplysina hirsuta* Hyatt; *Euspongia irregularis* L. J. Lendenfeld.

Reniera, *Pachychalina*, *Spinosella* and *Tedania* belong to the family of Haplosclerida; *Suberites* and *Donatia* are members of the families Suberitidæ and Donatidæ respectively; *Euspongia* and *Aplysina* are horny sponges belonging to the family Spongiidæ. The species of *Pachychalina* used in the experiments is characterized by its bright scarlet color. According to Brøndsted it does not belong to any of the previously described species.

The method employed in the present investigation was essentially the same as was used in previous work (Galtsoff, 1925). After rinsing the sponges in filtered sea water and removing the leaves of algæ and other foreign material, small pieces weighing 10 grams, were squeezed through bolting silk No. 20 into 100 ccm. of filtered sea water poured into a finger bowl or a crystallizing dish. Perfectly clean slides were placed on the bottom of the dish and the cells were allowed to settle on them. The slides were examined at regular intervals. For a study of agglutination, undiluted cell suspension of a given species was poured on a slide and mixed with a known amount of suspension of another sponge. The temperature of the water varied from 26° to 28° C.; the salinity of the water computed from hydrometer readings fluctuated from 29.5 to 31.5 parts per thousand.

Behavior of the Dissociated Cells.

Similar to the behavior of *Microciona* cells described by Wilson (1911) and Galtsoff (1925) the separated cells of each of the species of Bermuda sponges tested during the present investigation displayed amœboid movement and upon coalescence with each other formed aggregates. There was, however, a considerable difference in the velocity and duration of the amœboid movement of the cells of various species and, consequently, in the size and number of their aggregates. The most active amœboid movement resulting in the formation of a few large globular aggregates was displayed by the cells of *Reniera cinerea* and *Pachychalina*, whereas the cells of *Donatia* and *Spinosella* remained almost inactive and even after several hours following dissociation formed but very small and loose aggregates comprising a few cells. According to the ability of the separated cells to reunite, the sponges used in these experiments can be arranged in the following inequality series, beginning with the species which displayed the most active amœboid movement: *Pachychalina* sp. > *Reniera cinerea* > *Tedania ignis* > *Aplysina crassa* > *Aplysina hirsuta* > *Euspongia irregularis* > *Suberites carnosus* > *Donatia* sp. > *Spinosella*. The initial steps in aggregate formation by the dissociated cells of *Pachychalina*, *Reniera* and *Tedania* are similar to those observed in *Microciona*. The cells settle on the bottom forming a network of coalesced material, which gradually contracts and transforms into a number of round aggregates. During this period the archæocytes display an active amœboid movement forming large hyaline pseudopods and coalescing with one another and with other tissue cells, the amœboid movement of which is much slower. Unlike *Microciona* the cell aggregates of Bermuda sponges fail to form a firm attachment to glass or other solid surfaces (shells, rocks, celluloid, leaves of algæ). After a period varying from 12 to 24 hours they begin to curl up and detach themselves from the substratum. No further development has been noticed in the detached sphæroidal masses, which were kept for several days in the sea water; they all died within a few days. Similar difficulties were encountered by Wilson (1925) in his experiments

with several species of *Pachychalina* at Tortugas. De Laubenfels (1926, 1928) working on the same material, succeeded, however, in overcoming this obstacle, which he thinks was due to bacterial growth; he was able to keep the conglomerates of sponge cells at least two weeks, during which time they developed into functional sponges. Numerous attempts made in the course of the present investigation to prevent the detachment of aggregates by changing the pH of the water and by increasing the calcium content of it were unsuccessful. In spite of all efforts the separation of aggregates from the substratum occurred within 24 hours.

As has been stated above, the most active amœboid movement and aggregate formation occurs in the suspension of cells of *Reniera cinerea* and *Pachychalina*. *Reniera* suspension comprises a large number of archæocytes, 12–15 micra in diameter, loaded with large greenish granules. Less abundant are small round cells, 5–6 micra in diameter, containing few granules, and other cells of approximately the same size but entirely devoid of any protoplasmic inclusions. *Choanocytes* are very abundant and very active; by the beating of their cilia they agitate the suspended material and push around the cells which, coming in contact with the archæocytes, stick to their protoplasm and coalesce. In this manner the choanocytes play an important rôle in the reunion of separated cells. The aggregates of reunited cells continue to move over the substratum and coalesce with one another and with single cells that happen to lie on their route. The single archæocytes and the aggregates move in typical spiral paths (Galtsoff, 1923, 1925). In a few hours the amœboid movement ceases completely. By this time the coalesced cells have formed rounded aggregates, consisting of tightly packed cells, and have attached themselves to the substratum. The size and the number of aggregates found over a given area and formed under similar conditions can be used as a relative index of the duration and velocity of their movement. The slower the latter, and the shorter its duration, the greater is the number of aggregates and the smaller their diameter. In all the sponges tested, the process of the reunion of the separated cells is essentially similar to that just described, the only difference

being in the velocity and duration of the amœboid movement. It is therefore unnecessary to give a separate description for each species of sponge.

Heteroagglutination of Separated Cells.

Similar to the conditions found in a compound suspension of *Microciona* and *Cliona* cells the presence of foreign cells inhibits the amœboid movement of *Reniera* archæocytes and prevents their aggregation. This can be seen in the suspensions of *Reniera* and *Pachychalina* cells. The suspensions were prepared by squeezing 10 grams of sponge into 100 ccm. of sea water; 2 ccm. of suspension were added to 8 ccm. of sea water poured into Syracuse glasses. Various amounts of *Pachychalina* suspensions were added to *Reniera* suspension (Table 1). After three hours all the dishes were examined and the average number of aggregates for a given area (1.16 sq. mm.) was computed.

Table 1.

The Formation of Aggregates in a Compound Suspension of *Reniera* and *Pachychalina* Cells. (R—*Reniera*, P—*Pachychalina*. SW—sea water.)

Suspension.	Average Number of Aggregates.	Limits.	Number of Squares Examined.
2.0 R + 8.0 SW	7.9	5–12	20
2.0 R + 8.0 SW	9.6	4–16	20
2.0 P + 8.0 SW	5.0	3–7	20
2.0 P + 8.0 SW	5.9	3–9	20
2.0 R + 2.0 P + 6.0 SW	0		50
2.0 R + 1.0 P + 7.0 SW	0		50
2.0 R + 0.5 P + 7.5 SW	0		50

Microscopic examination of a compound suspension of *Reniera* and *Pachychalina* cells reveals that immediately upon the addition of foreign cells the amœboid movement of Reniera archæocytes is completely inhibited and that the beating of the flagella of *Reniera* choanocytes stops. The effect of *Reniera* cells on *Pachychalina* is even more pronounced; in a few seconds after Reniera cells are added to *Pachychalina* suspension the cells of the latter undergo rapid irreversible changes. The outer layer of the archæocytes bursts, the cells lose their regular shape;

the granuloplasm flows out through the injured part of the cell body and the cells undergo complete or partial cytolysis. This phenomenon is accompanied by an immediate agglutination of the suspended material, which forms large, loose flocculi settling on the bottom but failing to adhere to it. In less than half an hour the cells of *Pachychalina* are completely cytolized, while most of the *Reniera* cells remain alive but motionless. The agglutinated material perishes in a few hours.

The phenomenon of heteroagglutination occurs in several sponges and is essentially similar to that just described. It is most pronounced in *Reniera cinerea*, which agglutinates the cells of all other sponges tried in the present investigation. The results of all the experiments are summarized in table 2 in which the positive and negative agglutination reactions are denoted by + and − respectively. Several conclusions can be drawn from the examination of this table. It is shown that the heteroagglutination of separated cells is not always reciprocal. For instance, *Reniera cinerea*, which agglutinates the cells of all other sponges, is in turn agglutinated by *Tedania ignis* and *Aplysina crassa*, but is not agglutinated by *Pachychalina*. *Tedania* is agglutinated by *Aplysina hirsuta*, but the latter is not agglutinated by *Tedania*. *Pachychalina* agglutinates the cells of *Aplysina hirsuta* and is agglutinated by the latter; *Aplysina hirsuta* agglutinates *Pachychalina*, *Tedania* and *Spinosella; Euspongia irregularis* agglutinates *Spinosella* and is agglutinated by the latter; *Spinosella* agglutinates *Aplysina hirsuta* and *Euspongia irregularis* and is in turn agglutinated by *Euspongia* and *Aplysina hirsuta*. The determination as to which cells are agglutinated and which remain free is possible when the cells of two sponges can be recognized by their respective colors, or when the reaction is induced by the addition of a very small amount of agglutinating suspension. The formation of large flocculi and clarification of the turbid fluid can be observed with the naked eye.

In the majority of cases the reaction takes place immediately upon the addition of agglutinating suspension, but in a few instances from two to five minutes elapse before the formation of flocculi becomes noticeable.

The efficacy of the agglutinating suspension can be estimated by determining the minimum dose which causes the reaction. The differences in the agglutinability and agglutinating power of various sponges can be seen in table 3, showing the ratios between the minimum volumes of agglutinating and agglutinated suspensions involved in the reaction.

TABLE 2.

HETEROAGGLUTINATION OF DISSOCIATED SPONGE CELLS.

Sponge Added.	Sponge Agglutinated.								
	Reniera cinerea.	*Pachychalina* sp.	*Spinosella sororia.*	*Tedania ignis.*	*Donatia* sp.	*Suberites carnosus.*	*Aplysina crassa.*	*Aplysina hirsula.*	*Euspongia irregularis.*
Reniera cinerea	−	+	+	+	+	+	+	+	+
Pachychalina sp.	−	−	−	−			−	+	−
Spinosella sororia		−	−	−			−	+	+
Tedania ignis	+	−	−	−		−		−	−
Donatia sp.	−				−				
Suberites carnosus						−			−
Aplysina crassa	+	−	−	+			−	−	−
Aplysina hirsuta		+	+	+			−	−	
Euspongia irregularis		−	+	−			−	−	−

TABLE 3.

THE AGGLUTINABILITY OF VARIOUS SUSPENSIONS OF SPONGE CELLS.

Sponge Added.	Sponge Agglutinated.								
	Reniera cinerea.	*Pachychalina* sp.	*Spinosella sororia.*	*Tedania ignis.*	*Donatia* sp.	*Suberites carnosus.*	*Aplysina crassa.*	*Aplysina hirsula.*	*Euspongia irregularis.*
Reniera cinerea		1 : 25	1 : 10	1 : 50	1 : 25	1 : 25	1 : 25	1 : 25	1 : 20
Pachychalina sp.								1 : 5	
Spinosella sororia								1 : 10	1 : 10
Tedania ignis	1 : 5								
Aplysina crassa	1 : 5			1 : 5					
Aplysina hirsuta		1 : 5	1 : 1	1 : 1					
Euspongia irregularis			1 : 1						

As can be noticed from the examination of this table, the agglutination occurs in certain instances (*Reniera* vs. *Tedania*) when one drop of agglutinating suspension is added to fifty drops of agglutinable material, while in other cases (*Euspongia* vs. *Spinosella; Aplysina hirsuta* vs. *Spinosella*) equal volumes of two suspensions should be mixed to cause a positive reaction. It is interesting to note that *Reniera* agglutinates with almost equal readiness the cells of *Pachychalina*, which belongs to the same family of Haploscleridæ, and the cells of *Aplysinæ* and *Euspongia* belonging to a different order (Keratosa). On the other hand *Tedania ignis* is agglutinated more readily by *Reniera* than are the cells of the other genera of the family Haploscleridæ. Apparently the ability of sponges to agglutinate the cells of another genus cannot be correlated with their taxonomic position. Attention should be directed, however, to the fact that agglutination reaction fails to take place when the cells of two species of genus *Aplysina* are mixed together.

It has been found in the experiments with *Reniera* that the substance responsible for the agglutination of cells is soluble in sea water. The suspension of *Reniera* cells was filtered and centrifuged for 15 minutes at the rate of 1,200 revolutions per minute. The efficacy of clear supernatant fluid in inducing agglutination was equal to that of the unfiltered suspension.

It is known that, similar to the behavior of suspensions of inert particles, the suspensions of certain live cells (bacteria, red blood corpuscles) become unstable and agglutinate at the isoelectric point of the solution in which they are kept (Fauré-Fremiet et Nichita, 1927; Girard, 1912). On the other hand Coulter (1921), Eggerth (1924) and Winslow, Falk and Caulfield (1923) have shown that by acidifying the medium in which the cells are kept in suspension it is possible to neutralize the negative charges of the suspended particles. It is therefore possible to suppose that the agglutination in sponge cells may be due to the changes in the electric charges of the cells brought about by the changes in the hydrogen ion concentration of the surrounding medium. Crozier (1918), working on one of the Bermuda *Aplysinæ*, which he called Sponge A and which answers the description of *Aplysina crassa*, has demonstrated that the intracellular acidity of this

sponge is about pH 6.0. It has been noticed during the present investigation that the bright yellow color of the interior portion of the *Aplysina* tissues turns purple upon exposing it to sea water of the pH 8.2; the cells of the interior portion of the colony kept in acid sea water (pH 4.4–5.0) remain yellow. The conclusion seems inevitable that the tissues of *Aplysina crassa*, except those located on the surface of the sponge, are more acid than the surrounding sea water. Unfortunately other sponges used in the experiments do not possess pigments which can be used as indicators. In the light of work by Galtsoff and Pertzoff (1926), which has demonstrated that the suspensions of *Microciona* and *Cliona* cells behave either as weak bases or weak acids depending on the hydrogen ion activity of the suspension, it is reasonable to suppose that similar conditions exist in all siliceous sponges. One should expect therefore that the suspensions of sponge cells in sea water will decrease the pH value of the latter. As previous experiments have shown (Galtsoff and Pertzoff, 1926), the establishment of the new equilibrium between the cells and the surrounding medium takes a certain length of time and is dependent upon the number of cells in suspension. Several determinations of the pH values of the suspensions of Bermuda sponges, prepared under standard conditions (approximately equal number of cells in suspension; pH of sea water 8.2; temperature 26–28°), show that in ten minutes after the preparation of the suspension the reaction of the latter was acid (table 4).

TABLE 4.

PH VALUES OF THE SUSPENSIONS OF SPONGE CELLS.

	pH
Sea water	8.2
Reniera cinerea	6.4
Pachychalina sp.	6.0
Aplysina hirsuta	6.0–6.3
Aplysina crassa	6.6

Table 4 shows that there are no significant differences in the pH values of the suspensions which can be correlated with their agglutinating power and that apparently the agglutination of dissociated cells is not influenced by the increase in the hydrogen

ion concentration. Further support of this conclusion is found in the fact that the addition of 1/100 N HCl does not cause the agglutination of the suspended material. In this respect the dissociated sponge cells are similar to the amœbocytes of *Arenicola* and *Asterias*, the agglutination of which, as has been shown by Fauré-Fremiet (1927) is independent of the isoelectric point and above a certain lowest value occurs at any given pH. The conclusion can be reached that the agglutination of dissociated sponge cells is brought about by some substance or substances present in a suspension of cells, which cause the irreversible changes in the physical properties of the cell membrane resulting in breaking up of the cell body, efflux of the protoplasm, and aggregation of the suspended material. The nature of this substance is unknown.

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